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Temporal Regulation of Herpes Simplex Virus Type 1 *UL24*

W. JAMES COOK and DONALD M. COEN[1]

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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Using Northern blot, primer extension, and S1 nuclease analyses of wild-type and deletion-containing herpes simplex type 1 viruses, we found that *UL24* sequences are contained in six different transcripts that originate from three previously identified mRNA start sites. Thus, the six *UL24* transcripts represent three different pairs of 5′ coterminal mRNAs. Each transcript pair consists of a short species whose 3′ end corresponds to a polyadenylation signal located just downstream of the *UL24* open reading frame, and a longer species whose 3′ end corresponds to a polyadenylation signal located downstream of the *UL26* gene. Maximal accumulation of the short *UL24* transcripts was at early times during infection, while accumulation of the longer species did not decrease at late times. Consistent with early kinetics, the short transcripts were less sensitive to drugs that inhibited viral DNA replication than the longer transcripts which exhibited leaky–late kinetics. Quantitative S1 nuclease analysis indicated that 3′ ends corresponding to the *UL24* polyadenylation site were significantly more abundant at early times than at late times. Thus, differential polyadenylation determines the kinetics of accumulation of different *UL24* transcripts.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large double-stranded DNA virus whose 152-kbp genome predicts at least 70 protein coding genes (McGeoch *et al.,* 1988). HSV-1 genes are expressed in a temporally regulated fashion (Roizman and Sears, 1990; Wagner, 1994; Zhang and Wagner, 1987). Based on the timing of their expression and their dependence on viral DNA replication, HSV genes are categorized as immediate–early (α), early (β), or late (γ). Late genes can be further separated into leaky–late (γ1) and strict–late (γ2) depending on the onset of their expression and their sensitivity to drugs that inhibit viral replication. The mechanisms involved in controlling the temporal expression of early and late genes are poorly understood. While it is widely accepted that differences in transcriptional control due to variations in promoter architecture are the critical determinants of kinetic class (reviewed in Homa *et al.,* 1991), it remains possible that posttranscriptional regulation may also contribute to kinetic control (Blair *et al.,* 1987; Jones and Roizman, 1979; Kozak and Roizman, 1974; Singh and Wagner, 1993; Weinheimer and McKnight, 1987; Wobbe *et al.,* 1993). However, the details and impact of such posttranscriptional mechanisms for HSV are not understood.

Posttranscriptional mechanisms contribute to the transition from early to late gene expression in other DNA viruses including cytomegalovirus (Goins and Stinski, 1986; Stamminger *et al.,* 1991), adenovirus (Mann *et al.,* 1993; Nevins and Wilson, 1981), polyomavirus (Hyde-DeRuyscher and Carmichael, 1990; Liu *et al.,* 1994), and papillomavirus (Kennedy *et al.,* 1990; Kennedy *et al.,* 1991). In these examples, posttranscriptional effects include regulated RNA processing (Goins and Stinski, 1986; Hyde-DeRoysher and Carmichael, 1990; Kennedy *et al.,* 1990; Mann *et al.,* 1993; Stamminger *et al.,* 1991), altered RNA stability (Kennedy *et al.,* 1991), and antisense RNA control (Liu *et al.,* 1994).

We have been studying potential posttranscriptional mechanisms involved in expression of the HSV-1 *UL24* gene. *UL24* overlaps the 5′ end of the thymidine kinase (*tk*) gene (*UL23*) in a head-to-head orientation (Gompels and Minson, 1986; McGeoch *et al.,* 1988) (Fig. 1). The *UL24* open reading frame (ORF) is conserved among many herpesviruses that infect mammals and birds (Jacobson, 1992; Jacobson *et al.,* 1989). Disruption of the *UL24* ORF caused defects in viral growth (Jacobson *et al.,* 1989), suggesting that the UL24 protein is expressed and, although not essential, is important for HSV-1 replication in culture. Previous analyses of *UL24* mRNA expression (Fig. 1) detected three different 5′ ends for *UL24* transcripts (Kibler *et al.,* 1991; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980). There are two potential polyadenylation [poly(A)] sites for *UL24* transcripts: a proximal site located just downstream of the *UL24* ORF and a distal site at the end of the *UL26* gene (McGeoch *et al.,* 1988). However, previous Northern analyses detected only two transcripts containing *UL24* se-

[1] To whom correspondence and reprint requests should be addressed. E-mail: dcoen@warren.med.harvard.edu.

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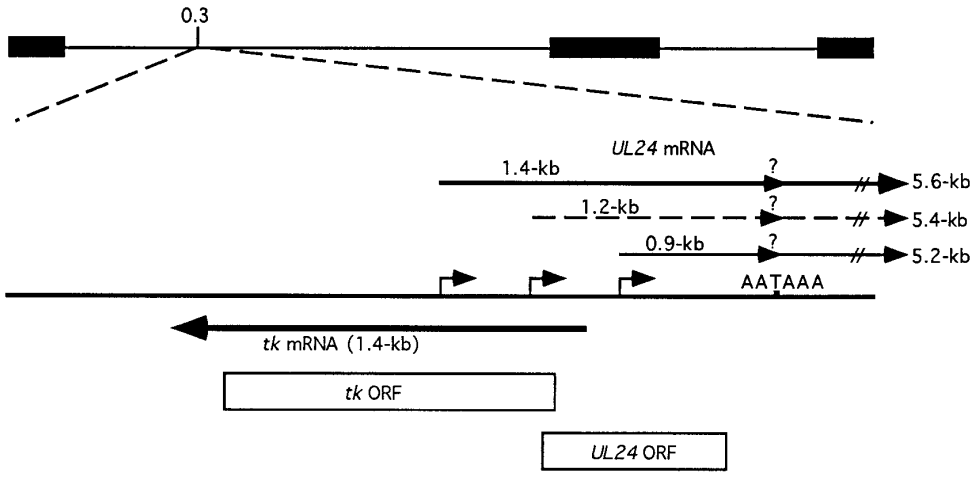

FIG. 1. A map showing the region of the HSV-1 genome which contains the *tk* and *UL24* genes, and reported and predicted transcripts produced in this region. The top line is a schematic representation of the HSV-1 genome in the prototype arrangement, with the location of *tk* and *UL24* at coordinate 0.3 indicated. Black rectangles represent major repeat sequences. The long line below the genome represents the DNA fragment containing *tk* and *UL24.* Shown on the DNA are the reported *UL24* mRNA start sites (Jacobson *et al.,* 1993; Kibler *et al.,* 1991; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980) and a potential *UL24* polyadenylation [poly(A)] sequence (AATAAA) (McGeoch *et al.,* 1988). Reported and predicted transcripts are shown above (*UL24*) and below (*tk*) the DNA fragment, with the thickness of transcript lines representing approximate relative abundance and arrowheads denoting 3′ ends. For *UL24* transcripts, the upstream arrowheads (labeled with question marks) represent termination at the potential *UL24* poly(A) signal. Short mRNAs which would be produced by use of this poly(A) signal are indicated by predicted sizes to the left of arrowheads; these transcripts have not been identified previously. Run-on through this poly(A) site to the downstream site results in the longer transcripts indicated by the sizes to the right. The 5.6- and 5.2-kb *UL24* transcripts have been described (Holland *et al.,* 1984). The 1.2- and 5.4-kb *UL24* transcripts, which are predicted based on identification of the mRNA start site from which these messages would originate, have not previously been detected on Northern blots and are therefore represented by a dashed line. Boxes representing the *tk* and *UL24* open reading frames (ORFs) are aligned at the bottom.

quences, a 5.6- and a 5.2-kb species (Holland *et al.,* 1984), whose 3′ ends appear to correspond to the distal poly(A) site (McGeoch *et al.,* 1988).

We report here that the transcriptional pattern and kinetics of accumulation of *UL24* mRNA are complex. We detected *UL24* sequences in six different transcripts that originate from the three previously identified mRNA start sites. The three short *UL24* mRNAs whose 3′ ends correspond to the proximal poly(A) signal were maximally expressed at early times during infection, while the longer *UL24* transcripts whose 3′ ends correspond to the distal poly(A) signal accumulated at peak levels throughout early and late times. Thus, the temporal expression of the different *UL24* transcripts is determined by differential polyadenylation.

MATERIALS AND METHODS

Viruses and cells

Viruses used were HSV type 1 strain KOS and the following mutants derived from it: PKG7 (Irmiere *et al.,* 1989), which is a control virus containing the PAA^{r}5 phosphonoacetic acid (PAA)-resistance mutation in the DNA polymerase gene and a temperature-sensitive *tk* mutation; LS-16/-6 (Coen *et al.,* 1986), which is a control virus containing the same mutations as PKG7 plus a linker scanning (LS) mutation in the *tk* promoter region (McKnight and Kingsbury, 1982); *dl*-197/-116 (Böni and Coen, 1989), which contains the same mutations as PKG7 plus a deletion of *tk* promoter sequences −197 to −116 (relative to the *tk* transcriptional start); ΔT143 (Irmiere *et al.,* 1989), which contains the PAA^{r}5 mutation plus a deletion of *tk* coding sequences +53 to +559; and *dl*sptk (Coen *et al.,* 1989), in which the only known mutation is a deletion of *tk* coding sequences +495 to +894. These viruses were propagated in Vero cells as described previously (Coen *et al.,* 1985). Viral DNA replication was inhibited as described (Wobbe *et al.,* 1993) by addition of 10 μg aphidicolin (Sigma) per milliliter for infections with PAA-resistant mutants, and 0.4 mg PAA (Sigma) per milliliter for KOS infections. Drug stocks were prepared as described (Chiou *et al.,* 1985).

Northern blot analysis

Vero cells were infected at a multiplicity of 10 PFU per cell, and isolation of total RNA and Northern analysis were performed as described (Cook *et al.,* 1995). Poly(A$^+$) RNA was isolated from total RNA using microcrystalline oligo(dT) cellulose (New England Biolabs) as described previously (Farrell, 1993). Preparation of radiolabeled, double-stranded DNA probes was described previously (Cook *et al.,* 1995). The *UL24* DNA probe was a ^{32}P-labeled, 550-bp *Eco*RI–*Hin*dIII fragment isolated from pLS/ts+5/+15 (Coen *et al.,* 1986) and contains sequences spanning the *UL24* ORF but not *tk* transcribed

sequences. The *tk* probe was a radiolabeled 660-bp *Sac*I–*Sma*I fragment (Coen *et al.,* 1986) containing *tk* coding sequences that do not overlap *UL24*. The *gB* DNA probe was a radiolabeled 900-bp *Pst*I fragment isolated from pSG18:SalE (Rafield and Knipe, 1984) that spans *gB* coding sequences. A riboprobe antisense to *UL24* was prepared by subcloning the *Eco*RI–*Hin*dIII *UL24* fragment into pBluescript (Stratagene), linearizing this plasmid with *Eco*RI, and transcribing a radiolabeled product using the Promega Riboprobe System as described by the manufacturer. Hybridization of the *UL24* riboprobe to Northern blots and subsequent washes were also as described by Promega.

S1 analysis

S1 nuclease mapping of the 3′ ends of *UL24* transcripts was performed as described previously (Baradaran *et al.,* 1994) using total RNA that was isolated as described (Cook *et al.,* 1995). The *UL24* S1 probe was prepared by subcloning a 2.7-kb *Eco*RI–*Sal*I fragment from pSG18 (Goldin *et al.,* 1981) into pBluescript, digesting this plasmid with *Ear*I, filling-in recessed ends with Klenow fragment and [α-^{32}P]dCTP (New England Nuclear) to add two radiolabeled nucleotides per 3′ end, and digesting with *Aat*II to liberate a 347-bp fragment [contains HSV-1 nucleotides (nt) 48499 to 48847; numbers are according to McGeoch *et al.* (1988) for the strain 17 sequence]. Under the conditions used, the intensities of signals corresponding to S1 nuclease cleavage at the 3′ ends of *UL24* mRNA were linear with the amount of RNA added (not shown). The *UL26* S1 probe was prepared by subcloning a 360-bp *Bam*HI–*Sac*II fragment from pSG18 into pBluescript, digesting this plasmid with *Afl*III, filling-in with Klenow fragment and [α-^{32}P]dCTP to add one radiolabeled nucleotide per 3′ end, and digesting with *Sac*II to liberate a 300-bp fragment (contains HSV-1 nt 52650 to 52950). Radiolabeled probes were purified from acrylamide gels as described previously (Cook *et al.,* 1995).

RESULTS

Characterization of *UL24* transcripts

Previous investigations identified three different 5′ ends of *UL24* transcripts using cytoplasmic and poly(A$^+$) RNA from HSV-infected cells or in transcripts generated *in vitro* using nuclear extracts (Jacobson *et al.,* 1993; Kibler *et al.,* 1991; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980; Wobbe, Jacobson, Yager, and Coen, unpublished; Fig. 1). These mRNA start sites were localized to positions corresponding to nt 47402, 47666, and 48076 in the HSV-1 strain 17 sequence (McGeoch *et al.,* 1988) (Figs. 1 and 2A). Previous Northern analysis detected *UL24*-specific transcripts of 5.6 and 5.2 kb that were predicted to share a 3′ terminus located downstream of *UL26* (Holland *et al.,* 1984), and therefore these transcripts appeared to be polycistronic messages containing *UL24*, *UL25*, *UL26*, and *UL26.5* mRNA (McGeoch *et al.,* 1988). However, McGeoch *et al.* (1988) noted a canonical poly(A) signal located just downstream of the *UL24* ORF that would predict an ~1.4-kb transcript originating from the nt 47402 start site, an ~1.2-kb transcript originating from the nt 47666 start site, and an ~0.9-kb transcript originating from the nt 48076 start site. Furthermore, a predicted ~5.4-kb transcript originating from the identified mRNA start site at nt 47666 and containing a 3′ end corresponding to the *UL26* poly(A) signal was not detected previously.

To address these remaining questions, we reexamined *UL24* mRNA expression. As an initial step to identify all possible *UL24* transcripts, Northern analysis was conducted on total RNA isolated from virus-infected Vero cells using a *UL24*-specific double-stranded DNA probe that does not overlap the transcribed region of *tk*. At 5 hr postinfection (p.i.), we readily detected three transcripts hybridizing to the *UL24* probe in LS-16/-6- and PKG7-infected cell RNA (lanes b and d, Fig. 2B). These transcripts included the previously identified 5.6- and 5.2-kb messages (Holland *et al.*, 1984), and a new transcript measuring 1.4 kb. A strand-specific *UL24* riboprobe hybridized to these same species, indicating that these transcripts were of the polarity expected for *UL24* (not shown). Upon longer exposures of autoradiographs, at 5 hr p.i. we could detect a 0.9-kb transcript (for example, see Fig. 5), and in some experiments a very faint 1.2-kb species (not shown). At 10 hr p.i., only the 5.6- and 5.2-kb transcripts were clearly visible in LS-16/-6 and PKG7 (lanes c and e, Fig. 2B). However, at 10 hr p.i. we observed a slight broadening of the 5.6-kb band toward the bottom of the gel, suggesting that a transcript that was slightly smaller than 5.6 kb was expressed later during infection. We predicted that this slightly smaller transcript was a 5.4-kb *UL24* species that originated from the start site at nt 47666. Previous studies showed that expression from this start site occurred at late times during infection (Read *et al.,* 1984; Wilkie *et al.,* 1980). The identity of the $\geqslant$6 kb RNA species most visible in samples harvested at 10 hr p.i. (lanes c, e, and g, Fig. 2B, and lanes b and d, Fig. 2C) is unclear; these transcripts may be readthrough mRNAs or transcripts originating from unidentified upstream start sites.

To help correlate the previously identified *UL24* mRNA start sites with transcripts identified on Northern blots, we conducted Northern analysis using viruses containing deletions that removed the reported *UL24* start sites. The *dl*sptk virus (Coen *et al.,* 1989) contains a deletion of *tk* coding sequences between nt 47054 and 47413 which was predicted to remove the most 5′ *UL24* promoter and start site at nt 47402 (Fig. 2A). Inspection of lanes f and g in Fig. 2B suggested that the 5.6- and 1.4-kb messages which should originate from this site were

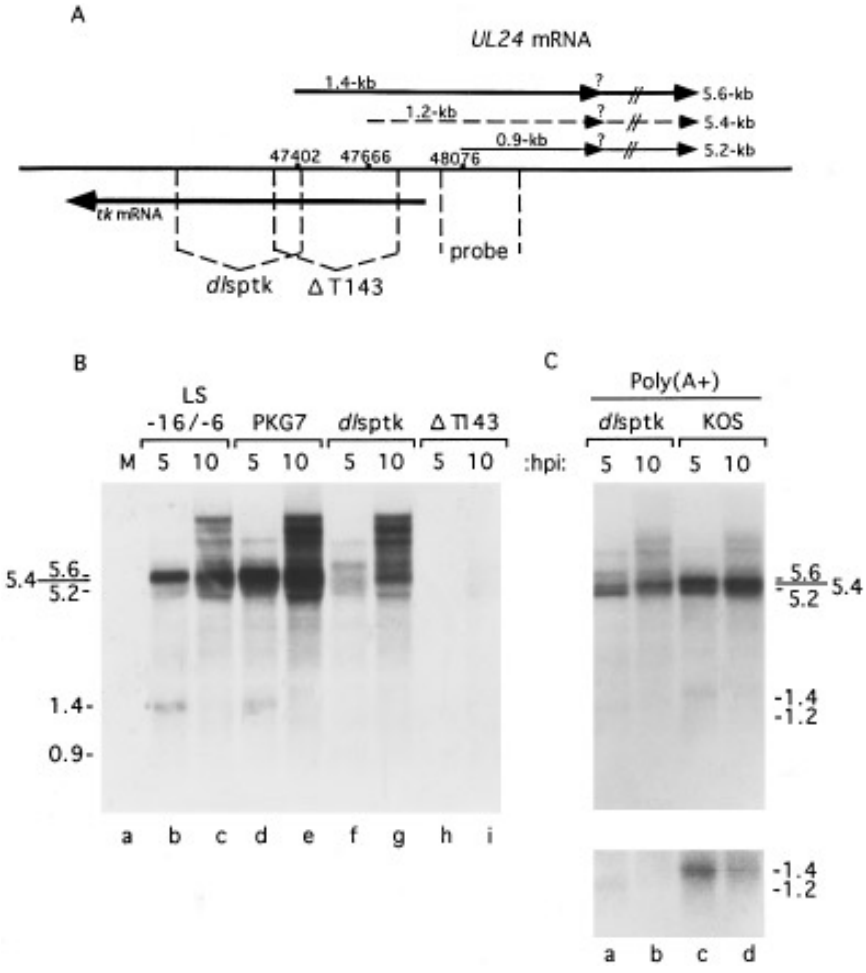

FIG. 2. Identification of *UL24* transcripts by Northern analysis. (A) Schematic diagram of the DNA fragment (represented by the long line in the middle) containing the *tk* and *UL24*, and *UL24* genes (top) and *tk* (bottom) transcripts produced from this fragment. Reported and predicted *UL24* transcripts are as described in Fig. 1. The locations of reported *UL24* mRNA start sites are indicated by HSV-1 nucleotide number based on the strain 17 sequence (McGeoch *et al.,* 1988) determined using references (Jacobson *et al.,* 1993; Kibler *et al.,* 1991; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980). The *UL24* DNA fragment used as a probe or as a template for a riboprobe is indicated by dashed lines, as are the DNA sequences deleted in the *tk* mutant viruses *dl*sptk and ΔT143. (B) Northern blot analysis of *UL24* mRNA was conducted on 5 μg of total RNA which was harvested at either 5 or 10 hr following infection of Vero cells with 10 PFU per cell with the indicated viruses or mock infection (M). The sizes of the transcripts hybridizing to the *UL24* probe were determined by stripping the Northern blot and reprobing for RNAs of known sizes including *tk*, *ICP8,* and *gB*, and by using 28S and 18S rRNA as size standards. (C) Northern blot analysis of *UL24* mRNA was conducted on poly(A^+) RNA purified from total RNA which was harvested as described in (B). The bottom panel is a longer autoradiographic exposure of the region of the blot containing the short *UL24* transcripts.

not present in RNA isolated from *dl*sptk-infected cells. However, we noted that resolution of the larger *UL24* transcripts was poor in Northern blots using total RNA, most likely due to deformation of these slow-migrating bands by 28S rRNA which migrates just below these signals.

To improve resolution of the long *UL24* transcripts and to determine if *UL24* mRNAs were polyadenylated, we purified poly(A^+) RNA from *dl*sptk- and KOS-infected cells and conducted Northern analysis as described above. In poly(A^+) KOS-infected RNA (lanes c and d, Fig. 2C), we detected the same *UL24* transcripts seen in LS-16/-6 and PKG7: the 5.6- and 5.2-kb species and the lower abundant 1.4-kb species. In poly(A^+) *dl*sptk-infected RNA, the 5.6- and 1.4-kb transcripts were clearly absent (compare lanes a and b to c in top and bottom panels, Fig. 2C). This result was in agreement with primer extension analysis in which mRNA originating from the *UL24* start site at nt 47402 was not detected in *dl*sptk-infected RNA (Wobbe and Coen, unpublished). Instead, in *dl*sptk we detected a 5.4-kb transcript that was maximally expressed at 10 hr p.i. (lane b, Fig. 2C) and a low-abundance 1.2-kb transcript that was faintly detected at 5 hr p.i. (lane a, Fig. 2C), in addition to the 5.2-kb transcript that was also present in the control viruses. Inspection of Figs. 2B and 2C and of other autoradiographs suggested that removal of the upstream promoter and start site in *dl*sptk caused overexpression of the 5.4-, 5.2-,

and 1.2-kb transcripts relative to that seen in the control viruses. Results shown in Fig 2C also indicated that each of the *UL24* transcripts is polyadenylated, with the possible exception of the 0.9-kb species. Furthermore, we observed the same kinetics of *UL24* mRNA accumulation using both poly(A^+) and total RNA preparations.

The ΔT143 virus (Irmiere *et al.,* 1989) contains a deletion of nt 47355 to 47861 which was predicted to remove the *UL24* start sites at nt 47402 and 47666 (Fig. 2A). Only the 5.2- and 0.9-kb transcripts were present in ΔT143-infected RNA (more visible upon longer exposure of the autoradiograph), while the 5.6-, 5.4-, 1.4-, and 1.2-kb transcripts were not detected (lanes h and i, Fig. 2B). Finally, we verified that the 5.2- and 0.9-kb transcripts originated from the start site at nt 48076 (Holland *et al.,* 1984; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980) by analyzing transcripts in the *dl*-197/-116 virus (Böni and Coen, 1989) which contains a deletion of nt 48030 to 48111 that removes this site. As predicted, the 5.2- and 0.9-kb transcripts were absent in *dl*-197/-116-infected RNA (not shown).

We inferred from these results that the 5.6- and 1.4-kb transcripts originate from the same start site at nt 47402 and thus are 5′ coterminal. This is supported by 5′ mapping studies (Jacobson *et al.,* 1993; Kibler *et al.,* 1991; Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980; Wobbe, Jacobson, Yager, and Coen, unpublished) that detected no other *UL24* mRNA start site 5′ to the *Sph*I site at nt 47413. Therefore, lack of the 5.6- and 1.4-kb transcripts in *dl*sptk strongly argues for the same promoter and start site driving these two transcripts. In addition, inspection of the 5.6- and 1.4-kb transcripts in Northern blots of infected-cell RNA from viruses containing deletions of nt 48030 to 48111 (*dl*-197/-116) and nt 47722 to 47922 (Δ1; Halpern and Smiley, 1984) revealed that these deletions removed sequences from both mRNAs (Cook and Coen, unpublished; Cook *et al.,* 1996). Our determination that the 5.4- and 1.2-kb transcripts both originate from the nt 47666 start site is in agreement with 5′ mapping data (Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980; Wobbe, Jacobson, Yager, and Coen, unpublished), and our determination that the 5.2- and 0.9-kb transcripts originate from nt 48076 agrees with 5′ mapping data (Read *et al.,* 1984; Read and Summers, 1982; Wilkie *et al.,* 1980) and Northern analysis (Holland *et al.,* 1984).

Mapping the *UL24* polyadenylation signal

We predicted that the 3′ ends of the 1.4-, 1.2-, and 0.9-kb *UL24* transcripts correspond to the canonical *UL24* poly(A) processing signal noted by McGeogh *et al.* (1988) which contains a consensus AATAAA sequence (Proudfoot and Brownlee, 1974) and a GT-rich sequence (Gil and Proudfoot, 1984; McLauchlan *et al.,* 1985) located 188 and 235 bp, respectively, downstream of the *UL24* ORF. We tested this by performing S1 nuclease mapping of the 3′ end of *UL24* using a radiolabeled probe that overlapped the putative *UL24* poly(A) site(Fig. 3). Three signals were detected using RNA isolated at 5 hr p.i. (early times) from infected cells. The strongest signal corresponded to the full-length probe and was consistent with the observation that substantial amounts of the 5.6-, 5.4-, and 5.2-kb messages were present in infected-cell RNA. The next strongest signal corresponded to a cluster of 3′ ends around nt 48767 which lies ~23 nt downstream of the AATAAA sequence and thus represents the terminus expected from processing at this poly(A) site. In addition, we detected a cluster of 3′ ends present

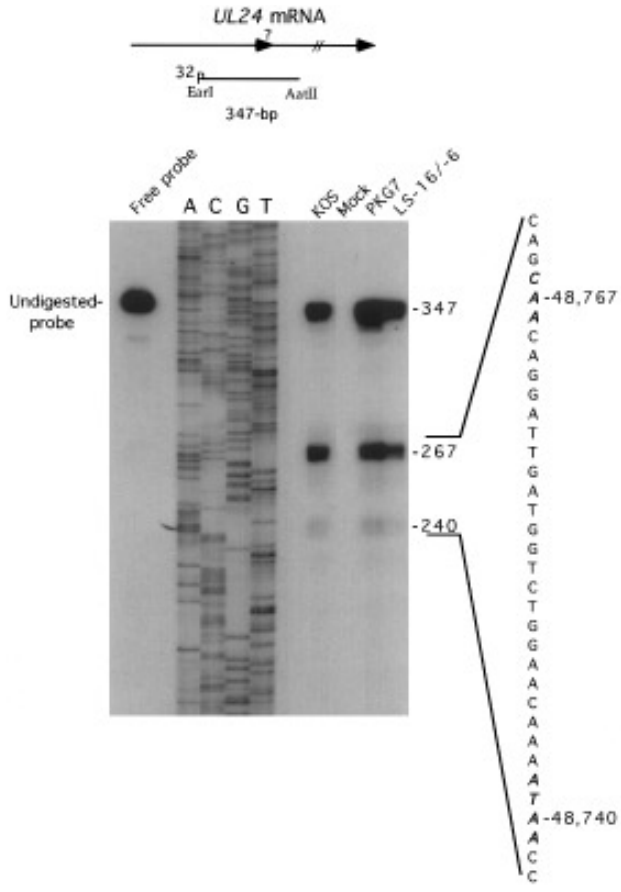

FIG. 3. S1 nuclease mapping of the 3′ ends of the short *UL24* transcripts. At the top the location of the 347-bp *Ear*I–*Aat*II probe used to map the 3′ ends of transcripts terminating at the *UL24* poly(A) signal is shown. The probe was hybridized to total RNA harvested at 5 hr following infection of Vero cells with 10 PFU per cell with the indicated viruses or mock infection . Following S1 nuclease digestion, radiolabeled protected DNA fragments were electrophoresed alongside sequencing reactions and undigested probe. Numbers immediately to the right of bands are in nucleotides deduced from the sequencing reactions and sequence information (McGeoch *et al.,* 1988). The strong signal that comigrates with undigested probe at 347 nt represents protection of full-length probe. The 267-nt signal corresponds to S1 cleavage ~23 nt downstream of the *UL24* AATAAA sequence as indicated by boldface and italicized letters in the DNA sequence shown to the right. The less intense bands at 240 nt correspond to cleavage within the AATAAA sequence as described in the text.

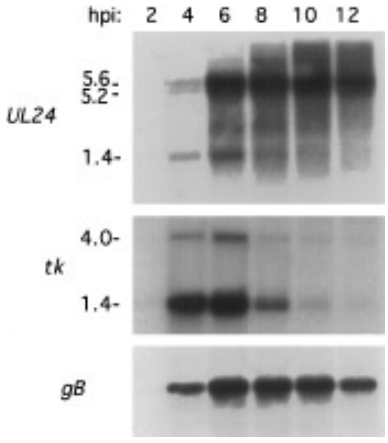

FIG. 4. The temporal expression of the 1.4-kb *UL24* transcript differs from that of the longer *UL24* transcripts. Northern analysis was conducted as described in Fig. 2, except that total RNA was isolated from Vero cells at different times following infection with LS-16/-6. The Northern blot was first hybridized with the *UL24* probe (top) and then successively stripped and reprobed for *tk* (middle) and *gB* mRNA (bottom). *UL24* mRNA levels in LS-16/-6 taken from this Northern blot are plotted as a function of time in Fig. 5B of Cook *et al.* (1996).

in low amounts that corresponded to cleavage within the AATAAA sequence around nt 48740. This S1-sensitive site was observed at low and variable levels in every experiment and may represent an artifact of the assay. These results indicate that the *UL24* poly(A) site is utilized during infection and that the 3′ ends of the 1.4-, 1.2-, and 0.9-kb transcripts are around nt 48767. The 3′ ends of the 5.6-, 5.4-, and 5.2-kb transcripts correspond to a site 4.2 kb downstream of the *UL24* poly(A) signal at the end of the *UL26* gene (Holland *et al.,* 1984).

The temporal expression pattern of the short *UL24* transcripts is different than that of the long *UL24* transcripts

Inspection of Fig. 2 suggested that the short *UL24* transcripts are maximally expressed early during infection, while accumulation of the long *UL24* transcripts does not decrease late in infection. We analyzed this further by measuring *UL24* mRNA expression in Vero cells infected with LS-16/-6 at different times postinfection (Fig. 4). *UL24* transcript levels in LS-16/-6 were quantified from the Northern blot, and values are plotted in Fig. 5B of Cook *et al.* (1996). Inspection of Fig. 4 indicated that levels of the 1.4-kb transcript in LS-16/-6 peaked at early times (i.e., 6 hr p.i.) and then decreased. This was identical to the kinetics of accumulation of the early (Harris-Hamilton and Bachenheimer, 1985; Sharp *et al.,* 1983) *tk* mRNA (Fig. 4). Upon longer exposures of autoradiographs, we noted that the 0.9-kb transcript also displayed an early temporal expression pattern similar to the 1.4-kb species (not shown). In contrast, abundance of the longer *UL24* transcripts did not decrease at late times, similar to that observed with the leaky–late (Homa *et al.,* 1988; Pederson *et al.,* 1992) *gB* mRNA (Fig. 4). Thus, the 1.4- and 0.9-kb *UL24* transcripts appeared to be expressed with early kinetics, while the longer *UL24* transcripts were expressed with leaky–late or late kinetics. While we could not detect the 1.2-kb transcript in this experiment, results shown in Fig. 2C suggested that this short message is also maximally expressed at early times.

The short *UL24* transcripts are less sensitive to inhibition of viral DNA replication than the long *UL24* transcripts

Maximal expression of HSV late genes, but not early genes, is dependent on viral DNA replication (Honess and Roizman, 1974; Roizman and Sears, 1990; Wagner, 1994). Therefore, drugs such as aphidicolin and PAA that inhibit viral DNA replication severely reduce or eliminate late gene expression, but have little or no effect on early gene expression. To test the effects of inhibiting viral DNA replication on *UL24* mRNA expression, we analyzed *UL24* mRNA accumulation in LS-16/-6 and PKG7 in the presence of 10 μg aphidicolin per milliliter (Fig. 5). Aphidicolin caused reductions in accumulation of each of the *UL24* transcripts; however, the levels of the 1.4- and 0.9-kb transcripts were much less affected by drug than the longer *UL24* transcripts. At 5 hr p.i. in both LS-16/-6 and PKG7, levels of the 1.4-kb transcript in the presence of aphidicolin were approximately 2-fold lower than in the no-drug controls (compare lanes c to a and g to e, Fig. 5). While we did not quantify the 0.9-kb transcript due to

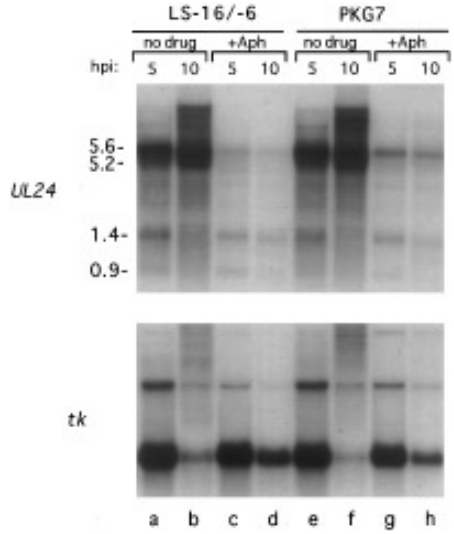

FIG. 5. Effects of aphidicolin on *UL24* mRNA levels. *UL24* mRNA accumulation was measured by Northern analysis as described in Fig. 2, except that infections were conducted either in the absence or in the presence of the DNA replication inhibitor aphidicolin at a concentration of 10 μg per milliliter. Following probing of the Northern blot for *UL24* mRNA (top), the blot was stripped and reprobed for the early *tk* mRNA (bottom) to control for loading and infectivity, and to show that early gene expression was not eliminated by excessive drug.

its low abundance, we observed that at 5 hr p.i. amounts of the 0.9-kb transcript were similar in both the absence and the presence of the drug (Fig. 5). In contrast, the longer *UL24* transcripts were reduced >10-fold in LS-16/-6 and >5-fold in PKG7 in the presence of aphidicolin. For comparison, levels of the early *tk* mRNA were not significantly reduced (<30%) in the presence of aphidicolin at 5 hr p.i. (Fig. 5, bottom). Similar patterns of *UL24* expression were measured during KOS infections in the presence of PAA (not shown). Thus, while expression of the 1.4- and 0.9-kb transcripts was only slightly reduced by replication-inhibiting drugs, accumulation of the longer *UL24* transcripts was substantially reduced. This result further indicates that the short *UL24* transcripts are expressed with different kinetics than the longer *UL24* messages.

Based on these results, we assign the 1.4-kb *UL24* transcript to the early kinetic class. While the low abundance of the 1.2- and 0.9-kb transcripts made their analysis more difficult, our results (Figs. 2C and 5) suggest that they too are expressed with early kinetics. The 5.6- and 5.2-kb transcripts were assigned to the leaky–late class, while the 5.4-kb transcript was assigned to the strict–late class based on its later onset of accumulation as detected in the *dl*sptk virus (Fig. 2) and data from other references (Read *et al.,* 1984; Wilkie *et al.,* 1980). Because of difficulty in distinguishing the 5.6- and 5.4-kb transcripts on Northern blots, we considered the possibility that we might have incorrectly attributed accumulation of the 5.4-kb mRNA to the 5.6-kb transcript at late times and therefore wrongly assigned the 5.6-kb mRNA to the leaky–late class. However, our designation of the 5.6-kb mRNA as leaky–late is consistent with previous work which showed that the primer extension product representing the 5.6- and the much less abundant 1.4-kb transcripts clearly displayed leaky–late kinetics of accumulation (Jacobson *et al.,* 1993; Kibler *et al.,* 1991; Wobbe and Coen, unpublished).

3′ Ends corresponding to the *UL24* poly(A) site are more abundant at early than late times

The results outlined above suggested that the early temporal expression of the 1.4-, 1.2-, and 0.9-kb *UL24* transcripts results from utilization of the proximal *UL24* poly(A) site at early times, but decreased utilization of this poly(A) signal at late times during infection. To address this further, we performed quantitative S1 mapping of the 3′ ends of the *UL24* mRNAs using a radiolabeled 347-bp *Ear*I–*Aat*II probe that overlaps the 3′ end of *UL24* (Fig. 6). A radiolabeled 300-bp *Afl*III–*Sac*II probe that overlaps the 3′ end of *UL26* was used as a control.

Inspection of Fig. 6 revealed the same major S1-protected species using the *UL24* probe as seen in Fig. 3: the 347-nt species corresponding to protection of full-length probe presumably by the long *UL24* transcripts,

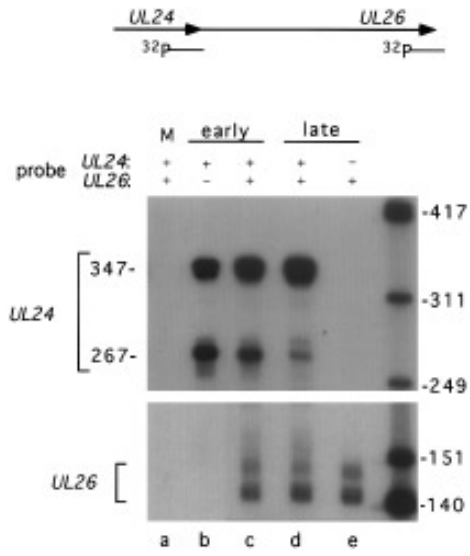

FIG. 6. Quantitative S1 mapping of *UL24* 3′ ends at early and late times postinfection. Shown at the top are the locations of the 347-bp *UL24* probe described in Fig. 3 and the 300-bp *Afl*II–*Sac*II probe which overlaps the *UL26* poly(A) signal. Hybridization of probes to total RNA, S1 digestion, and electrophoreses were as described in Fig. 3. In all samples 20 μg of total RNA from LS-16/-6-or mock-infected cells was used. Lane a, mock-infected RNA hybridized to the *UL24* and *UL26* probes; lane b, infected-cell RNA harvested at 5 hr p.i. hybridized to the *UL24* probe; lane c, infected-cell RNA harvested at 4 hr p.i. hybridized to the *UL24* and *UL26* probes; lane d, infected-cell RNA harvested at 10 hr p.i. hybridized to the *UL24* and *UL26* probes; lane e, infected-cell RNA harvested at 10 hr p.i. hybridized to the *UL26* probe. The 347- and 267-nt *UL24*-specific signals (top) and the 142- to 147-nt *UL26*-specific signals (bottom) are indicated. Markers (in nt) are from *Hin*fI-digested øX154 DNA.

and the 267-nt species corresponding to processing expected at the proximal *UL24* poly(A) signal. We compared the intensity of the 267-nt signal to two controls: the signal due to full-length protection of the *UL24* probe (347 nt), and a signal obtained with the 300-bp *UL26* probe which produced a cluster of bands measuring 142 to 147 nt that corresponded to processing at the *UL26* AAUAAA sequence. Inspection of lanes c and d in Fig. 6 showed that the intensity of the 267-nt *UL24* species was 7.0-fold higher at 4 hr p.i. than at 10 hr p.i. In contrast, the intensity of the 347-nt *UL24* signal was 1.5-fold lower at 4 hr p.i. than at 10 hr p.i., and the intensity of the *UL26*-specific signal was 1.7-fold lower at 4 hr p.i. than at 10 hr p.i. Thus, the levels of 3′ ends corresponding to utilization of the *UL24* poly(A) signal are maximal at early times postinfection, while the signals representing *UL24* readthrough transcripts and 3′ ends of transcripts ending at the *UL26* poly(A) signal are slightly higher at late times. These results are consistent with processing at the *UL24* poly(A) site being more efficient at early times than at late times postinfection.

DISCUSSION

In this report, our examination of *UL24* mRNA expression revealed that the kinetics of *UL24* mRNA accumula-

tion are determined by differential polyadenylation. This could be due to differential utilization of poly(A) sites or, less simply, to differential transcript stability following poly(A) addition. We compare this with other examples of kinetic control resulting from differential polyadenylation in HSV and other viruses and discuss the potential biological significance of this regulation.

Kinetic control via differential polyadenylation

Differential utilization of poly(A) signals is important for determining temporal expression of genes in cytomegalovirus (Goins and Stinski, 1986; Stamminger *et al.,* 1991), adenovirus (Mann *et al.,* 1993; Nevins and Wilson, 1981), polyomavirus (Hyde-DeRuyscher and Carmichael, 1990), and papillomavirus (Kennedy *et al.,* 1990). In HSV-1, a poly(A) site in the end of the *UL2* gene was reported to be more efficiently used at early than at late times during infection, and this resulted in the shorter of two 5′ colinear transcripts accumulating with leaky–late kinetics while the longer transcript accumulated with strict–late kinetics (Singh and Wagner, 1993). These findings resemble those shown here for *UL24*, except that the short and long *UL24* transcripts exhibit early and leaky–late kinetics compared to the leaky–late and strict–late kinetics observed for the *UL2*-containing transcripts. Furthermore, the apparent efficiencies of utilization of the *UL24* poly(A) signal appear to be less than that for the *UL2* poly(A) signal based on the figures in Singh and Wagner (1993). However, the use of different experimental conditions makes it difficult to compare our results directly with those of Singh and Wagner.

A comparison of the *UL24* and *UL2* poly(A) signals that appear to be inefficiently utilized at late times with the *UL26* signal that appears to be efficiently utilized late indicated that each of these signals contains the consensus AATAAA sequence and potential downstream GT-rich motifs [consensus YGTGTTYY (Y = pyrimidine)] (McLauchlan *et al.,* 1988). However, subtle sequence variations in the GT-rich sequences are evident. For example, the *UL24* GT-rich sequence is located 41 bp downstream of the AATAAA tract which is a wider distance than found in the *UL26* signal where the distance is 26 bp. The potential GT-rich sequence in the *UL2* poly(A) signal, GGGTCGGGTG, deviates from the consensus more than the *UL26* signal and, like the *UL24* GT-tract, is located further downstream (33 bp) of the AATAAA. It has been reported that poly(A) site usage in certain genes was decreased when the distance between the AATAAA signal and the GT-rich tract was increased beyond 40 bp (Brown *et al.,* 1991; reviewed in Wahle, 1995). However, sequence comparisons of HSV poly(A) sites from early and late genes did not reveal obvious sequence patterns that might differentiate them (McLauchlan *et al.,* 1989).

Previous *in vitro* analyses have identified an activity found in extracts of HSV-infected cells [originally designated late processing factor (LPF) that selectively increases processing at poly(A) sites for certain transcripts (McLauchlan *et al.,* 1989, 1992). The HSV immediate–early regulatory protein ICP27, which is involved in the switch from early to late gene expression (McCarthy *et al.,* 1989; Rice and Knipe, 1990), is required for full induction of LPF (McLauchlan *et al.,* 1992). ICP27 stimulates expression of genes containing certain poly(A) sites (Brown *et al.,* 1995; McLauchlan *et al.,* 1992; Sandri-Goldin and Mendoza, 1992; Smith *et al.,* 1992) and binds to the 3′ ends of certain mRNAs (Brown *et al.,* 1995). Interestingly, one ICP27-responsive poly(A) signal contained a nonconsensus GT-rich sequence located 42 bp downstream of the AATAAA, while certain nonresponsive signals contained consensus GT-rich tracts located within 30 bp of the AATAAA (Sandri-Goldin and Mendoza, 1992). These results suggest the possibility that ICP27 stimulates processing of inefficient poly(A) signals such as that of *UL24* at early times during infection, but this stimulation is turned off at late times. Alternative explanations for inefficient use of certain poly(A) signals late during infection include greater competition for the cellular polyadenylation/cleavage machinery (Mann *et al.*, 1993) and the activity of a specific viral protein at late times that represses utilization.

Potential biological relevance

The unusual regulation of the kinetics of *UL24* mRNA accumulation may have no biological significance to the virus. However, we can envision certain scenarios by which the virus might benefit from this regulation. Low-level expression of the short *UL24* transcripts may be beneficial to the virus at early times but deleterious late. The possible importance of modulating levels of the short *UL24* transcripts is supported by findings in the accompanying report (Cook *et al.,* 1996) where we show that accumulation of the 1.4-kb transcript at early times is down-regulated by *tk* expression. Also, constant low-level expression of the long transcripts at both early and late times might be beneficial. For example, because the long transcripts are polycistronic for *UL24, UL25, UL26,* and *UL26.5,* regulated processing at the *UL24* poly(A) signal might be important for decreasing expression of these downstream genes at early times. Assessment of the full biological significance of regulated *UL24* polyadenylation will require further experiments including analyses of the expression and function of UL24 protein.

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